Histone Deacetylase Inhibition and Blockade of the Glycolytic Pathway Synergistically Induce Glioblastoma Cell Death

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Abstract

Purpose: High-grade gliomas are difficult to treat due to their location behind the blood-brain barrier and to inherent radioresistance and chemoresistance.

Experimental Design: Because tumorigenesis is considered a multistep process of accumulating mutations affecting distinct signaling pathways, combinations of compounds, which inhibit nonoverlapping pathways, are being explored to improve treatment of gliomas. Histone deacetylase inhibitors (HDIs) have proven antitumor activity by blocking cell proliferation, promoting differentiation, and inducing tumor cell apoptosis.

Results: In this report, we show that the HDIs trichostatin A, sodium butyrate, and low nanomolar doses of LAQ824 combined with the glycolysis inhibitor 2-deoxy-D-glucose induce strong apoptosis in cancer cell lines of brain, breast, and cervix in a p53-independent manner. HDIs up-regulate p21, which is blocked by concomitant administration of 2-deoxy-D-glucose.

Conclusions: We propose simultaneous blockade of histone deacetylation and glycolysis as a novel therapeutic strategy for several major cancers.

Glioblastoma multiforme (GBM) are the most frequent human brain tumors. These aggressive, highly invasive, and neurologically destructive tumors are among the deadliest of human cancers, with a median survival ranging from 9 to 12 months (1). Despite treatment efforts including new technological advances in neurosurgery, radiation therapy, and clinical trials with novel therapeutic agents (2), median survival has not changed significantly over the past two decades. Meanwhile, cancer drug development has been moving from conventional cytotoxic chemotherapeutics to more sophisticated drugs exploiting biological mechanism of tumorigenesis (3).

In high-grade gliomas, several genes and pathways are altered due to a severe mutator phenotype that leads to the accumulation of mutations in critical regulatory genes. Mutations of PTEN, RB, p16/p14, p53 (4), and receptor tyrosine kinase (5–7) result in up-regulation of pathways that promote tumor growth, invasion, and resistance to apoptotic stimuli (8).

Besides classic mutations, epigenetic silencing of tumor suppressor genes frequently leads to dysregulation of signaling pathways promoting tumorigenesis. Histone acetyltransferase and histone deacetylase (HDAC) catalyze the acetylation and deacetylation of lysine residues in the tails of histone proteins (e.g., lysine residue in histones 3 and 4), regulating the affinity of the protein transcriptional complexes to the DNA (9). Thus, the recruitment of histone acetyltransferase and HDAC is considered a key element in the dynamic regulation of genes involved in cellular proliferation and differentiation during normal development and carcinogenesis (10). HDAC inhibitors (HDI) induce re-expression of silenced tumor suppressor genes (11, 12). HDIs induce differentiation and promote apoptosis in transformed and cancerous but not in normal cells (11, 13). HDIs have been classified in different classes: short-chain fatty acid (as sodium butyrate, valproic acid, etc.), Epoxides (as depudecin and trapoxin), cyclic peptides, Benzamides, and Hydroxamic acids [as trichostatin A (TSA), SAHA, and LAQ824]. Besides the chemical TSA, sodium butyrate (NaB), and SAHA (Vorinostat; Food and Drug Administration approved in 2006), a new potent cinnamic hydroxamic acid derivate, LAQ824 (13), is currently used in clinical trials for the treatment of leukemia (14–16). Because this drug passes the blood-brain barrier, it may potentially be useful for the treatment of malignant gliomas. In vitro treatment of tumor cell lines with the LAQ824 caused hyperacetylation of histones, restoring the expression of <2% of human genes, including the cell cycle kinase inhibitor p21WAF1/Cip1 (17). High concentrations of LAQ824 induce accumulation in G2 phase of the cell cycle and are selectively toxic for transformed and cancer cells, whereas lower concentrations induce cell cycle arrest in G1, increase p21 expression, and only weakly induce apoptosis (18). The presence or the absence of p21 seems to play a critical role for the commitment of the targeted cell to either undergo growth arrest or apoptosis in response to LAQ824.

Maintenance of growth and proliferation of tumor cells requires high cellular energy levels. Tumor cells have abnormal mitochondrial functions and essentially rely on glycolysis to provide ATP for their metabolic requirements even under...
aerobic condition (Warburg effect). As a consequence, tumor cells have relatively low oxygen needs and can survive in a hypoxic environment that is not suitable for normal cells. Human malignant gliomas were shown by 18 F-fluoro-2-deoxyglucose positron emission tomography studies to be much more avid of glucose than the normal cortex (19). This high energy demand of cancer cells is the rational basis to block glycolysis in cancer cells (20). Glucose metabolism and ATP production are inhibited by the glucose analogue 2-deoxy-D-glucose (2-DG). Upon phosphorylation by hexokinase, 2-DG selectively accumulates in cancer cells by increased glucose transporter expression, high hexokinase activity, and low phosphorylase activity (21, 22). 2-DG strongly reduced growth of tumor cells, when used as single agent (23). Moreover, 2-DG impairs repair of radiation-induced DNA damage in tumor cells and promotes tumor cell apoptosis by lowering intracellular energy levels (24). In the treatment of human GBM, 2-DG is used as a radiosensitizer in combination with radiotherapy (24, 25). Moreover, at least two clinical trials using 2-DG for solid tumor and intracranial metastases are ongoing.

Because HDIs induce re-expression of tumor suppressor genes in several distinct pathways and cancer cells critically depend upon continuous energy supply, we explored the therapeutic potential of a combination of two compounds that simultaneously target the epigenetic status and the energy demand of cancer cells. Synergistic effects may even allow to reduce the drug

Fig. 1. Synergistic induction of apoptosis by 2-DG and HDAC inhibitor LAQ824 in glioma cell lines. Glioma cell lines Hs683, LN401, SF767, U343 and U373 (A), nontumor lines HEK and rat aorta smooth muscle cell, and normal rat astrocytes (B) were precultured for 48 h in standard medium and were exposed to various concentrations of LAQ824 in the presence of 2-DG for 72 h. Apoptosis was determined by FACS analysis. The CI calculated for the combination of 25 mmol/L 2-DG and 60 nmol/L LAQ824 were 0.085 (Hs683), 0.066 (LN401), 0.018 (SF767), 0.053 (U343), and 0.009 (U373), respectively. CIs lower than 1 indicate synergy in all cases (see the Materials and Methods section for cutoffs).
dosage of potentially toxic HDIs (26). We found that the combination of 2-DG and HDIs lead to a strong synergistic effect resulting in widespread apoptosis of tumor cells of the brain, breast, and cervix, while sparing normal rat astrocytes.

Materials and Methods

Cell lines and reagents. Hs683, LN401, SF767, U373, U343 glioma cell lines, for which the genetic status of established cancer genes TP53, p16/p14, and PTEN has been defined (4), were cultured in Eagle medium supplemented with 25 mmol/L glucose, glutamine, standard antibiotics, and 10% FCS. HCT116, HeLa, MCF-7, HBL, and SKBR-3 cells were cultured in RPMI containing 25 mmol/L glucose, glutamine, standard antibiotics, and 5% FCS. Parental p21+/+ and engineered p21-/−HCT116 (27) were a generous gift from Prof. Bert Vogelstein (Johns Hopkins University, Baltimore MD). Fresh rat astrocytes were cultured as described (28). All cells were maintained at 37°C in 5% CO2. All cell lines were seeded in 3-cm plates at 35% density and grown for 48 h before treatment with the indicated drugs. LAQ824, RAD001, and Imatinib (Gleevec) were kindly provided by Novartis-Pharma AG. 2-DG, TSA, and NaB were purchased from Sigma (Saint-Louis). Drug concentrations described are indicated in the figures.

Western analysis and antibodies. Cells were washed with 1× PBS; lysed in a buffer containing 2% SDS, 50 mmol/L Tris (pH 6.8), and 0.1 mol/L DTT; boiled; and either used immediately or frozen at -20°C. Protein lysates were resolved on denaturing SDS-polyacrylamide gels ranging from 8% to 13% and transferred to nitrocellulose membranes (Hybond; enhanced chemiluminescence; Amersham Biosciences). Membranes were probed with the following primary antibodies: anti-Akt (kindly provided by Dr. Brian Hemmings, Friedrich Miescher Institute Basel, CH); anti-S6 (kindly provided by Dr. George Thomas, Friedrich Miescher Institute Basel, CH); antibodies against phosphorilated S6 protein (Ser-240/244), S6K and phospho-S6K (Ser-389), phospho-Akt (Ser-473), and poly(ADP)ribose polymerase (PARP) were purchased from Cell Signaling; and antibodies against p21, Cyclin A, extracellular signal-regulated kinase (p42 and p44), and Phospho–extracellular signal-regulated kinase (Tyr-204 of p42 and p44) were purchased from Santa Cruz Biotechnology. Decorated proteins were revealed using horseradish peroxidase–conjugated anti-mouse or anti-rabbit immunoglobulins (New England Biolabs) and visualized by enhanced chemoluminescence (Amersham Biosciences).

Cell viability assay. Cell DNA content and apoptosis were analyzed with a Flow cytometer (FACS Calibur; BD Biosciences), and statistics were determined with Cell Quest software. Cells were trypsinized and fixed in ice-cold 70% ethanol for 1 h, stained with 50 μg/mL propidium iodide for fluorescence-activated cell sorting (FACS) analysis. The percentage of dead cells was determined by the proportion of cells in pro-G1 phase. Percentages reported result from three independent experiments.

Combinatorial index calculation. Apoptosis resulted from the combination of two drugs was analyzed for each cell line using Fig. 2. Synergistic induction of apoptosis of 2-DG and HDAC inhibitor LAQ824 in nonglioma cell lines. HeLa (cervix) and HBL, MCF-7, and SKBR-3 (breast) cancer cell lines were precultured for 48 h in standard medium and were exposed to drugs at various concentrations for 72 h. Apoptosis was determined by FACS analysis. The CI calculated for the combination of 25 mmol/L 2-DG and 60 nmol/L LAQ824 were 0.05 (HeLa), 0.4 (HBL), 0.1 (MCF-7), and 0.6 (SKBR-3), respectively, indicating synergy in all cases.
CalcuSyn Software (Biosoft) to generate a combination index (CI). A CI greater than one indicates antagonism. A CI of one indicates an additive effect. A CI less than one indicates synergism. Specifically, a value between 0.85 and 0.90 indicate slight synergism, between 0.70 and 0.85 indicates a moderate synergism, between 0.30 and 0.70 indicates synergism, between 0.10 and 0.30 indicates strong synergism, and <0.1 indicates a very strong synergism.

**Cell proliferation assay.** Cells ($5 \times 10^3$) were seeded in 96-well dishes and grown for 8 h before treatment. Cells were then exposed to 60 nmol/L LAQ824 and 25 mmol/L 2-DG for 24 h. Bromodeoxyuridine incorporation was allowed for the last 2 h. Cell proliferation Biortak Elisa system (Amersham Biosciences) was done according to manufacturer’s instructions. Experiments were done in triplicate. Data were analyzed with GraphPad prism4 software (GraphPad Software Corporation).

**ATP measurement.** Cells cultured in 6-well dishes were treated with 25 mmol/L 2-DG and 60 nmol/L LAQ824, lysed, and incubated at room temperature for 5 min. ATP levels were measured using a luciferase-based assay kit (ATP Bioluminescence Assay kit CLS II; Roche). For each well, ATP levels were normalized by comparison with the amount of total protein by the Bio-Rad protein assay reagent. All ATP level measurements and protein assays were done in triplicate.

**Results**

Blocking glycolysis strongly enhances apoptosis induced by HDAC inhibition. In malignant glioma cells, we targeted the histone acetylation pathway with the HDI LAQ824, a known inducer of cell death in tumor and immortalized cell lines, and blocked the energy pathway with the glucose analogue 2-DG. Increasing amounts of LAQ824 and 25 mmol/L 2-DG were applied to 5 glioma cell lines during 72 hours of treatment, and apoptosis was monitored by FACS analysis (Fig. 1A). The use of
LAQ824 alone induced relatively low levels of apoptosis in a concentration-dependent manner in all glioma cell lines tested, whereas 2-DG alone triggered cell death just above baseline. However, the combination of LAQ824 and 2-DG significantly enhanced cell death in all lines tested. The extent of synergism was dependent on the concentration of both drugs but predominantly on the concentration of 2-DG. The observed synergism was found to be independent of the p53 status because p53–wild-type SF767 and U343 and p53-mutant Hs683, LN401, U373 tumor cells responded in a similar way (Fig. 1A). The noncancer cell lines HEK293, rat aorta smooth muscle cells, and rat astrocytes do not show an enhanced cell death upon combining 2-DG with LAQ824 (Fig. 1B).

To test whether this synergism is also observed in other tumor types, we tested the 2-DG/LAQ824 combination in breast and cervix cancer cells MCF-7, HBL, SKBR-3, and HeLa. We found that these epithelial cancer cells exposed to 2-DG together with increasing doses of LAQ824 exhibited the same synergistic induction of cell death as we had consistently observed in a panel of glioma cells (Fig. 2).

To establish whether the synergistic induction of apoptosis triggered by the association of LAQ824 and 2-DG represents a general mechanism not limited to the drug LAQ824, we used other HDIs such as TSA, used as an antifungal antibiotic, and the chemical compound NaB, currently used in clinical trials, in combination with 2-DG. Consistent with the results obtained with LAQ824, the association of 2-DG with TSA or NaB strongly induced apoptosis in glioma and cervical cancer lines (Fig. 3A and B).

Titrations established that concentrations of 25 mmol/L 2-DG and 60 nmol/L LAQ824 on Hs683 cells were optimal for synergy (Fig. 1A). Using these conditions, apoptosis started as early as 16 hours after treatment and constantly increased until 72 hours, although application of each drug alone induced little or no cell death at these concentrations (Fig. 3C). The appearance of the cleaved 86 kDa form of PARP at 16 hours indicated the onset of apoptosis induction resulting from the combined treatment. Only low levels of cleaved PARP appeared after 36 hours of 25 mmol/L 2-DG treatment alone, and no evidence for cleavage was present upon LAQ824 application alone (Fig. 3D).

2-DG and LAQ824 affect cell cycle regulation. Next we studied the effect of LAQ824 on Hs683 and HeLa cell cycle as single agents or combined with 2-DG. After 24 hours of treatment, both 2-DG and LAQ824 reduced Hs683 and HeLa proliferation rate (Fig. 4A). We also analyzed the effect of 2-DG and LAQ824 on G1 and G2 phases of cell cycle. 2-DG and LAQ824 as single agents induced G1 and G2 accumulation, respectively, whereas the combination had minimal effect on G1 and G2 distribution but strongly inhibited the S phase of the cell cycle (Fig. 4B).

ATP levels and S6 phosphorylation are affected by 2-DG. Decreased ATP levels lead to the activation of the energy sensing kinase AMP-protein kinase. Its activation blocks the high energy consuming mechanisms by reducing the activation of S6K and phosphorylation of the ribosomal protein S6 at serine residues 240 and 244, thereby blocking global protein translation. To establish whether synergistic induction of apoptosis by 2-DG and/or LAQ824 involves the energy
regulatory pathway, ATP levels and the S6 protein phosphorylation status were studied. Upon application of 25 mmol/L 2-DG, a dramatic decrease in ATP levels was measured while the presence of LAQ824 did not alter ATP levels (Fig. 4C). A comparable decrease was observed by combining both drugs (Fig. 4C). We observed a decrease in intracellular phosphorylated S6 protein in cells treated with 2-DG, whereas the application of LAQ824 did not alter the phosphorylation of S6 protein (Fig. 4D). Reduction of phosphorylated S6 protein levels were also observed in HeLa cells line after 2-DG treatment (data not shown). These results show that 2-DG dramatically inhibits the energy regulatory pathway and may considerably block translation by reducing the amounts of phosphorylated S6 protein.

2-DG abrogates p21 expression induced by LAQ824. We then investigated the role of p21 in the synergistic induction of apoptosis by following levels of p21 protein in the glioma cell line Hs683 after addition of the HDI LAQ824. Starting 4 hours after LAQ824 treatment, p21 levels showed a transient prominent increase dropping again to undetectable protein levels at 48 hours (Fig. 5A). When 2-DG and LAQ824 were applied together, the p21 increase disappeared (Fig. 5B). To test whether lowering 2-DG concentrations would reduce p21 protein levels, intermediate doses of 2-DG were combined with 60 nmol/L of LAQ824. After 8 hours of treatment, p21 levels were significantly augmented in presence of LAQ824 and reduced with increasing doses of 2-DG (Fig. 5B). Consistent increase of p21 levels upon addition of LAQ824 alone and its disappearance after 2-DG treatment was observed in HeLa cells (Fig. 5B) and all other glioma cell lines tested (data not shown). Moreover, as with LAQ824, the HDIs TSA and NaB caused an increase in p21 protein levels that was strongly reduced in the presence of 2-DG both on the glioma line Hs683 and the cervix cancer line HeLa (Fig. 5C).

Lack of p21 protein sensitizes cancer cells to apoptosis. To test whether the 2-DG effect on p21 protein is a critical factor in the synergistic induction of apoptosis, we tested the effect of the HDI LAQ824 on the parental colon cancer cell line HCT116, which is wild-type for p21 (p21+/+), and its p21−/− derivate (27). HCT116 p21−/− cells were very sensitive to LAQ824, undergoing rapid apoptosis. Nevertheless, HCT116 p21−/− cell line showed a 15% to 20% increase of apoptosis compared with the wild-type constellation HCT116 p21+/+, both after 24 hours and after 72 hours of application of the HDI LAQ824 (Fig. 6A). Combination of LAQ824 and 2-DG on HCT116 p21+/+ and p21−/− cells did not change the different apoptosis rates observed (Fig. 6B). These data show that the reduction of p21 protein levels may increase apoptosis rate upon treatment with the HDI LAQ824.

Synergistic induction of apoptosis in fresh ex vivo GBM cell cultures after the combined treatment of 2-DG with LAQ824. Because commonly used cell lines undergo many cycle divisions with a high probability to increase their genetic mutator phenotype, we tested LAQ824 and 2-DG in combination on an ex vivo GBM-derived cell line BS153. BS153 cell line showed a synergistic response upon LAQ824/2-DG combination when compared with the single drug application (Fig. 6C). Thus, targeting the energy and the epigenetic pathways may be an efficient treatment also in ex vivo GBM cell lines.

LAQ824 specifically synergizes with 2-DG but not with the rapamycin derivative RAD001 or Imatinib. We tested the specificity of the synergy between LAQ824 and 2-DG by measuring apoptosis of glioma cells Hs683, U87, and U373 in response to LAQ824 in combination with other compounds that target alternative nonoverlapping glioma pathways such as the mammalian target of rapamycin inhibitor RAD001 or the platelet-derived growth factor inhibitor Imatinib. Neither combination increased the apoptosis rate in GBM cell lines (Fig. 6D), showing the specificity of synergy between combined
Discussion

Combination of drugs that inhibit nonoverlapping cancer pathways is a rational strategy to control tumor growth. A synergistic effect after the use of two drugs can lower the single dosage and increase the patient tolerance, reducing collateral effects. We have shown that 2-DG, an inhibitor of the glycolytic pathway, sensitizes glioma cells treated with HDI to undergo apoptosis. Epigenetic silencing of tumor suppressor genes leads to dysregulation of cellular processes promoting tumorigenesis. It is well-established that 2-DG and HDIs such as Vorinostat (butyrates) are able to pass the blood-brain barrier, a prerequisite for glioma treatment, and are also well-tolerated by patients. We found that HDIs such as TSA or NaB strongly synergize with 2-DG in the induction of tumor cell death. We then tested LAQ824, an HDI that is currently in clinical trial against leukemia and displays good blood-brain barrier penetration, for its potential as a cancer drug against malignant glioma. In response to higher nanomolar doses of LAQ824, glioma cell lines underwent apoptosis, whereas lower nanomolar doses only had a cytostatic effect with markedly reduced cell death. However, a combination of 2-DG and low nanomolar concentrations of LAQ824 showed a marked synergism in the induction of tumor cell apoptosis, not only in GBM cells, but also in breast and cervix cancer cells, regardless of their p53 status. The observed synergism seems to be specific because combination with other drugs that target glioma pathways such as RAD001 and Imatinib did not lead to any increased apoptosis rate when combined with LAQ824.

The glycolysis demand of many tumors represents the rationale to block glucose metabolism in addition to histone deacetylation inhibition. We first tested the effect of 2-DG per se on human GBM cell line survival, which induced apoptosis in GBM cells only at high concentrations (25 mmol/L) but...
efficiently inhibits proliferation of all glioma cell lines. Glycosylation of essential proteins such as transcription factors can be profoundly affected by the absence of glucose (29) or by blocking glycolysis with 2-DG (30). Under conditions of energy starvation, the decreased ATP to AMP ratio activates the first cellular energy sensor, the tumor suppressor LKB1 (31), and the Tuberous Sclerosis Complex TSC1/2 (32) activate 5′AMP-protein kinase. Activation of AMP-protein kinase leads to a reduction of global translation and cell size by inhibiting phosphorylation of the downstream effectors such as the mammalian target of rapamycin (33), the S6 40S ribosomal kinase protein kinase, and the eukaryotic initiation factor 4E binding protein. 2-DG attenuates the transcription factor activity of Sp1 by influencing its O-GlcNAcylation levels (30). Because many transcription factors are modified by O-GlcNAc, it is likely that O-GlcNAcylation of other transcription factors could regulate gene expression in response to glucose use.

HDI are able to restore the expression of 2% of the total number of human genes (17), e.g., the cyclin-dependent kinase inhibitor p21 whose up-regulation is considered to be responsible for the antiproliferative effect of the drug (17, 34). A number of studies have shown that p21 expression causes G1 cell cycle arrest and promotes cell cycle arrest and apoptosis of cancer cells (18, 35, 36). However, among cells that have HDI-induced p21 expression, only cells in late G1 and S phase proceed through an aberrant mitosis and rapid apoptosis, whereas cells in G1 phase accumulate p21 and undergo cell cycle arrest. Our results show that LAQ824, TSA, and NaB applied alone induced a transient increase of p21 protein levels that is completely abolished upon addition of 2-DG, which then drives the cells into apoptosis.

Other drug combinations (37), e.g., cisplatin and the rapamycin derivative RAD001 (38), the HSP90 antagonist 17-AAG, and NaB (39, 40), can induce similar synergism regarding the implication of p21 proteins in the regulation of cell survival. We therefore conclude that the p21 response to some drug combinations including HDIs is not the causative factor of induction of tumor cell apoptosis because marked decrease of p21 levels also accompanied induction of widespread tumor cell death in the combination of 2-DG with HDI. Nonetheless, p21 background slightly influences cell death response upon drug application, as found when exposing p21−/− and p21−/− knockout cancer cells.

In conclusion, we propose to develop a clinical protocol that explores the therapeutic potential of a combination of 2-DG and LAQ824 for glioblastoma that can be extended to other malignancies such as breast and cervix cancer.

**Disclosure of Potential Conflicts of Interest**

Novartis covered 25% of all expenses and 75% of noncommercial expenses of the research.

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